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J. Parasitol. 30:295-302, 1944.

J. Protozool., 8:410-416, 1961.

J. Parasitol. 73:311-326, 1976.

Poultry Sci. 54:2081-2086, 1975.

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Methods in coccidiosis research: separation of oocysts from faeces

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SUMMARY

Factors which may be important in the large-scale extraction of coccidial oocysts from faeces have been investigated with *Eimeria tenella*. Age of bird, inoculum, feeding status at the time of inoculation, period of collection, feeding status during collection, collection medium, homogenization and sieving, flotation, washing, sporulation and further purification have all been considered. The aim has been to establish a method to produce the maximum number of oocysts of a required degree of purity and viability, with the expenditure of the minimum amount of physical effort, time, animals and chemicals. In our method, groups of chickens 3–4 weeks of age are inoculated with 5000 oocysts of *E. tenella* and food is supplied *ad lib*. Over the period 5–8 days after inoculation, faeces are collected in trays containing 2% (w/v) potassium dichromate solution, while food intake is restricted. The faecal material is homogenized, passed once through 40 and 100 mesh sieves, centrifuged and the oocysts recovered from the sediment by 3 flotations in saturated salt solution. Following washing, oocysts are sporulated by forced aeration at 30 °C and may be further purified by hypochlorite treatment, or passage in 5% Tween 80 solution through a glass bead column followed by sucrose density gradient centrifugation. Routine passages along these lines over a 5 year period have given a recovery of 46% of the oocysts excreted by over 7000 birds.

INTRODUCTION

In any programme of coccidiosis research, it is necessary to isolate oocysts from infected tissues or faeces as a source of infective material or an object of study; the degree of oocyst purity required will depend on the use to which the material will be put. Although isolation from intestinal tissues may have some advantages when only small numbers of oocysts are required, such preparations will contain many immature and non-viable oocysts, and far larger yields of mature oocysts per animal will be obtained from faecal preparations. For isolation from faeces, Davies, Joyner & Kendall (1963) have briefly described typical laboratory methods involving sieving and/or salt flotation, while Edgar (1964) patented a large-scale method involving repeated washing and settling in dichromate solution. Although all conventional methods will yield some oocysts, few authors other than Vetterling (1969) have been concerned with the *efficiency* of the method employed.

In a large-scale drug screening and evaluation programme, and in biochemical investigations of oocysts and sporozoites, where large amounts of material are required, the efficiency of the oocyst production method is of paramount importance. We have started with a conventional *ad hoc* routine used in these laboratories for many years, and critically investigated each step of the procedure. The aim throughout has been to produce the maximum number of oocysts of a required degree of purity and viability, with the expenditure of the minimum amount of physical effort, time, animals and chemicals. In our programme of work, economy in manpower is particularly important. The methods evolved we believe to be the best compromise between the numerous, often opposing, operating constraints. The investigations have been carried out with *E. tenella*, but should indicate the sort of considerations to be taken into account during the isolation of other species.

EXPERIMENTAL

The Houghton isolate of *E. tenella* was used to infect Thornber 288 White Leghorn type cock chicks fed a commercial broiler mash specially compounded to be deficient in vitamin K and free from antibiotics or anticoccidial agents. (The vitamin K deficiency is not regarded as necessarily desirable in oocyst production work, but is advantageous for the drug-screening programme we operate. Its effect is to enable the production of a desired degree of pathology with a smaller inoculum of the haemorrhagic species than would be required using a complete diet, i.e. results in an economy of material required as inoculum. The deficient diet was used here to avoid duplication of diets in our overall programme of work.) Our choice of bird has been governed by availability of a plentiful and cheap supply of discard cockerels from a hatchery geared to pullet production. For oocyst counts, samples were diluted at least 10-fold with saturated NaCl solution, and oocysts counted in a McMaster worm egg slide specially ruled with 12 channels (instead of the usual 6) per 1 cm square so that the chamber could be used with a $\times 10$ objective and $\times 10$ eyepieces (Hawksley, Lancing, Sussex). In the case of faecal counts, faeces were homogenized for 10 min with approximately 4 volumes of water; triplicate 1 ml samples of the homogenate were removed while still being mixed, and diluted with 9 ml saturated salt solution for counting. Duplicate counts were carried out on each sample.

Infection

Inoculation of birds with small numbers of oocysts allows a relatively large production of oocysts in terms of numbers inoculated (Hein, 1968; Ryley, Millard & Long, 1972). Large inocula may result in the death of birds before oocyst production starts, or at least sufficient parasitization and destruction of intestinal epithelium by the earlier asexual stages to prevent optimal sexual development. Bigger birds will have greater areas of intestinal epithelium to support coccidial development, but will require more effort in husbandry, and will be at risk of adventitious coccidial infection longer than smaller birds. We seek to define the

Table 1. T

Deaths/total inoculated

Total oocysts/ surviving bird (millions)

Oocyst yield (oocysts produced / oocysts inoculated) (in thousands)

Oocyst yield (millions/g chick infected)

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Table 1. *The effect of age of bird and size of inoculum on oocyst yield with* Eimeria tenella

	Age (days)	Oocysts inoculated 10250	5125	2560	1280	640	320	Av. wt at inoculation (g)
Deaths/total inoculated	4	8/20	0/20	1/20	0/20	2/20	1/20	50
	10	10/20	10/20	4/20	5/20	4/20	3/20	51
	15	9/20	4/20	2/20	0/20	1/20	0/20	89
	21	1/15	2/15	0/15	0/15	1/15	0/15	117
	30	7/10	2/10	0/10	2/10	2/10	3/10	260
	39	0/10	0/10	0/10	0/10	0/10	0/10	349
Total oocysts/ surviving bird (millions)	4	34·5	42·7	33·8	35·5	26·9	13·3	
	10	49·8	57·0	50·4	48·2	38·3	20·8	
	15	64·1	78·7	69·5	67·7	54·7	57·0	
	21	201·6	145·5	113·4	157·6	66·6	63·8	
	30	143·2	193·7	149·5	105·7	112·9	80·9	
	39	195·1	143·9	118·7	100·3	79·3	48·8	
Oocyst yield (oocysts produced / oocysts inoculated) (in thousands)	4	3·36	8·34	13·18	27·70	42·00	41·48	
	10	4·85	11·10	19·61	37·60	59·80	65·20	
	15	6·25	15·36	27·13	52·90	85·50	178·3	
	21	19·63	28·40	46·25	122·9	104·1	199·3	
	30	14·00	37·80	58·40	82·40	176·5	252·5	
	39	19·05	28·10	46·30	80·00	123·8	152·6	
Oocyst yield (millions/g chick infected)	4	0·69	0·85	0·67	0·71	0·54	0·26	
	10	0·99	1·12	1·00	0·96	0·76	0·41	
	15	0·72	0·89	0·79	0·77	0·62	0·65	
	21	1·73	1·25	1·02	1·35	0·57	0·55	
	30	0·55	0·75	0·58	0·41	0·44	0·31	
	39	0·56	0·41	0·34	0·29	0·23	0·14	

Oocyst outputs measured over days 4–10.

optimal inoculum and age of bird to produce the biggest yield of oocysts, with the least effort and greatest economy in chicks. Feeding status at the time of infection may influence the speed of passage of coccidia through the intestine, and degree of contact between sporozoites and the intestinal epithelium.

Age of bird and size of inoculum

In the experiment summarized in Table 1, groups of 10, 15 or 20 chickens of various ages were inoculated (day 0) with a series of doubling dilutions of sporulated oocysts of *E. tenella*. Daily oocyst counts were carried out for each group from days 4 to 10 inclusive. It will be seen that with most inocula, more oocysts were produced in birds 30 days of age than in older or younger ones, although yields were quite reasonable over ages 21–39 days. In terms of oocysts produced per gram of chicken inoculated, the best yields were obtained in the 10–21-day age range. We consider that with our conditions and strain of bird, 3–4 weeks of age is optimal for oocyst production. At this age, actual output was more or less constant ($100–200 \times 10^6$ per bird) over an inoculum range of $1–10 \times 10^3$, although in relation

Table 2. *The effect of starvation before inoculation on oocyst output*

Experiment	Age of bird (days)	Inoculum	Oocyst yield (millions/chick) Normal	Starved
1	10	500	18·7	21·2
	10	5000	53·7	54·8
	24	500	26·7	50·6
	24	5000	94·8	112·8
2	10	500	10·1	16·1
	10	5000	27·4	31·0
	24	500	26·8	37·0
	24	5000	60·7	48·7
3	10	5000	63·2	66·5
	24	5000	136·9	177·8

Oocyst output per bird measured over days 5–9 inclusive.

to oocysts inoculated, the yield decreased approximately 5-fold and mortality became a problem at the top end of this range. A satisfactory inoculum for birds aged 3–4 weeks would seem to be $1–5 \times 10^3$.

Feeding status at time of inoculation

Edgar (1964) suggested that a more uniform infection with a bigger percentage take results from starvation of chicks for several hours prior to inoculation. Groups of thirty 10-day-old or fifteen 24-day-old chicks were inoculated with 500 or 5000 sporulated oocysts as indicated in Table 2, duplicate groups being fed normally or starved for 24 h prior to inoculation. Total oocyst output was measured over days 5–9. In most cases, starvation before inoculation resulted in a bigger oocyst yield, but the effect was extremely variable, with a stimulation of oocyst output from 2 to 90%. Starvation before inoculation may be recommended, but is not critical.

Collection

Period of collection

Different strains of coccidia show variation in the timing of their life-cycle. Oocyst outputs were measured daily in all groups in the experiment summarized in Table 1. The daily counts for all inocula were added together, and expressed as a percentage of the total count for all days and all inocula; these daily outputs are represented diagrammatically in Fig. 1. Oocyst output will doubtless continue beyond day 10, but there is a level of output where it is no longer profitable to expend physical effort to isolate just a few oocysts. The output for days 5–8 represents 87·3% of the total, and we currently consider it worthwhile with our strain to process the faeces excreted over this period. Separate experiments indicated equally efficient oocyst recoveries and sporulation rates whether faecal collections in dichromate were worked up daily, or whether the total collection for days 5–8 was worked up in one batch.

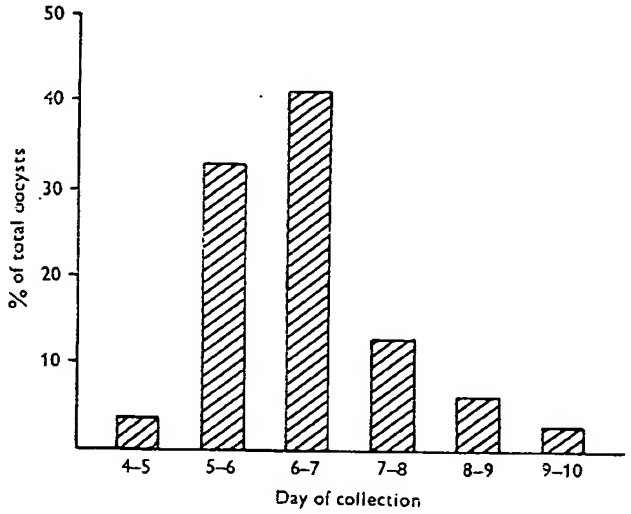

Fig. 1. Oocyst output during *Eimeria tenella* infection.

Collection medium

Faeces for oocyst isolation may be collected into liquid, or dry. Drying out may impair oocyst viability, and certainly makes it more difficult to prepare the homogeneous suspension necessary for the subsequent isolation procedure. For years we have collected into water. When we started to investigate oocyst recovery at the salt flotation stage (see later section), it became apparent that although oocysts should float in saturated salt solution, many, and in some cases the majority, were being carried down mechanically with the unwanted faecal debris. Thus in 10 batches of sieved, water-collected faecal suspension, oocyst recoveries varied from 10·5 to 65·5%, with an average of 38·5%. The majority of the oocysts lost could be found in the *sediment* at the salt flotation stage, even though up to 4 successive flotations were in some cases carried out. It seemed probable that faeces collected into water were undergoing extensive decomposition even though collections were processed daily, and that bacterial growth accompanied by the production of mucilagenous material was responsible for binding oocysts to the grosser debris. Subsequent experiments (Table 3) confirmed the inefficiency and *variability* of the extraction procedure, but indicated that consistently higher oocyst recoveries could be achieved by acidification to pH 1 or by collecting into potassium dichromate solution. Acidification of faeces to pH 1 was accompanied by evolution of gas, and a smell so revolting that some technicians were unwilling to carry out the isolation procedure! Oocyst recoveries were depressed under alkaline conditions.

Feeding status during collection

Restricting food during the collection period could conceivably result in the production of less faeces with less feed contamination, but no appreciable cut in

Table 3. *Effects of collection medium and pH on % oocyst recoveries at the salt flotation stage*

Experiment	pH	Collection medium: Water	Collection medium: Dichromate
1	1·0	110*	–
	6·5	80	–
	10·0	54	–
2	1·0	103	–
	2·0	80	–
	2·7	93·5	–
	3·8	49	–
	5·0	82	–
	5·7	76	–
3	1·0	80	81
	5·0	–	84
	6·5	35	–
	11·0	–	16
4	1·0	95	87
	5·0	–	92·5
	6·5	87	–
5	1·0	92	95
	5·0	–	86
	6·5	73	–
6	5·0	–	97
	6·5	39	–

Faeces collected into water (pH 6·5) or 2% (w/v) potassium dichromate (pH 5·0) and oocysts extracted by 2 salt flotations.

* % oocyst yields.

oocyst output, so leading to a more efficient and less arduous extraction process. Seven experiments were carried out in which 2 groups of thirty 3-week-old chicks were given the same inoculum of oocysts, but when faeces were collected over days 5–8, one group was fed continuously, while feeding in the other was restricted. This was achieved either by removing the collecting tray and supplying food *ad lib.* for 1 h per day, or by supplying 13 g of food per bird per day without removing the collecting tray. On an average, birds with restricted food intake during collection produced only 20–25% of the bulk of excrement produced by birds fed continuously. The total oocyst output of the birds on restricted diet was rather surprisingly only 85% of that of the continuously fed birds, so that concentration of the oocysts was not as marked as expected. When worked up by identical methods, slightly fewer oocysts present in faeces from continuously fed birds were recovered, compared with those from birds on restricted feeding.

Table 4. *R*

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Table 4. *Recovery of oocysts after repeated sieving of faecal suspensions in dichromate*

Homogenization	Sieving no.	% recovery 20 mesh	40 mesh	100 mesh
No	1	–	71·4	88·9
	2	–	13·1	8·1
	3	–	6·9	1·0
	Residue	–	10·3	1·4
No	1	89·1	92·5	88·0
	2	10·3	4·0	6·7
	3	3·4	0·4	0·4
	Residue	5·4	0·6	7·5
Yes	1	–	93·9	98·2
	2	–	4·3	1·5
	3	–	0·4	0·1
	Residue	–	0·2	0·0

Preliminary treatment

Homogenization and sieving

Conventional methods usually employ hand-operated or mechanical sieves or muslin to remove coarser faecal debris prior to oocyst isolation, in order to reduce the amount of material to be processed in subsequent stages. We have used 8 in. diameter 40 and 100 mesh sieves (apertures approx. 390 and 150 μm respectively). Coarse faecal clumps were broken on the sieve by means of a 3 in. wide paint scraper. Counts before and after sieving soon showed that appreciable numbers of oocysts could be retained on the sieves, trapped mechanically by coarser debris. Further oocysts could be recovered by resuspension of the solid fraction in water and further sieving. In the first 2 experiments recorded in Table 4, batches of 5 l dichromate-collected faeces containing 3×10^9 oocysts were put through a series of sieves, oocyst counts being carried out on the filtrate and residue at each stage. Debris retained on sieves was resuspended in water and resieved, each sieve being used 3 times before the combined filtrates were subjected to the finer sieve. In the third experiment, an 8 l batch of dichromate-collected faeces was homogenized for 15 min with an overhead Vortex mixer (Peter Silver, Hampton, Middlesex; model LA type 2) before being sieved. Such a mixer quickly achieves adequate comminution of faecal debris, and is more convenient than a conventional blender in that it can be used in a bucket with large quantities of material. Preliminary homogenization not only made sieving easier, but a greater percentage of oocysts passed through the sieve at the first attempt. Although repeated sieving or the use of greater dilutions resulted in the recovery of more oocysts, it also increased the volume of material to be subsequently centrifuged.

As a result of these and similar experiments, we have adopted as standard procedure a 3 min period of homogenization followed by one passage through a 40 and one through a 100 mesh sieve.

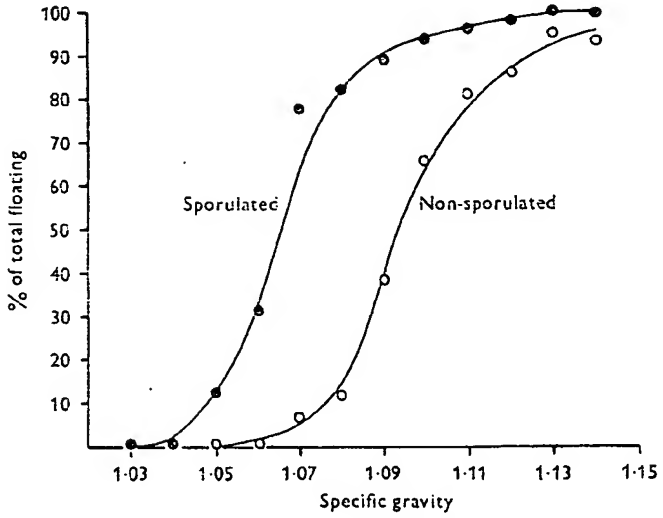

Fig. 2. Buoyancy of *Eimeria tenella* oocysts.

Sporulation

Some workers prefer to sporulate oocysts before separation from faecal material while others leave the process until after isolation. Vetterling (1969) found his method resulted in a preferential isolation of the lighter, sporulated oocysts from an incompletely sporulated population. Faeces from infected chicks were collected in ice and a crude oocyst preparation isolated as quickly as possible. This was then purified on a glass bead column with 5% Tween 80 as described later. Half the material was sporulated in dichromate, the remainder being kept refrigerated. Approximately 5×10^6 oocysts of each type were pipetted into a series of tubes and sedimented. Sucrose solutions (20 ml) of various densities (determined by hydrometer) were added to the tubes, the oocysts suspended, and the tubes then centrifuged. Oocysts were counted in the scum which formed on the surface, in the supernatant and in the sediment. Fig. 2 indicates the percentage of the total population which floated at any given specific gravity (sp. gr.). From this it might be expected that oocysts would be easier to separate by flotation if they were sporulated first. This is not necessarily the case, since the majority of oocysts, sporulated or not, floated at a sp. gr. of 1·13, and saturated salt which we routinely use, has a sp. gr. of 1·20; saturated salt solution can be diluted to 66% saturation before a sp. gr. of 1·13 is reached.

A 3 l batch of faecal suspension in dichromate was aerated at room temperature using a 2 cm sintered glass disk on the air line; 56% sporulation was achieved in 24 h, but was no more than 58% at 72 h. Only 49% of the oocysts present were extracted by the normal procedure, compared with 92% of the oocysts in another sample of the same batch which was not aerated prior to extraction. This latter culture reached 70% sporulation in 72 h after isolation. Further experiments indicated a consistently lower sporulation rate when aeration was carried out

Table 5. (

First spin:
Second spin:

Third spin:

Figures give % rec
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Table 5. *Comparison of salt, zinc sulphate and sucrose solutions as flotation media*

		Flotation medium (sp. gr. 1·20)		
		NaCl	$ZnSO_4$	Sucrose
First spin:	Recovery	87·6	75·9	70·2
Second spin:	Recovery	80·5	73·7	84·6
	Cumulative recovery	97·2	93·8	95·6
Third spin:	Recovery	83·5	80·5	69·0
	Cumulative recovery	99·8	98·8	98·3

Figures give % recoveries by flotation of oocysts present during 3 successive centrifugations and cumulative % recovery.

prior to oocyst isolation, and a consistently less efficient extraction of oocysts. Forced aeration of the faecal material was accompanied by the growth of micro-organisms, particularly filamentous fungi and yeasts, in spite of the presence of 2% potassium dichromate. It is assumed that this microbial growth was responsible for the poor sporulation and extraction achieved. Attempts were made to reduce microorganismal proliferation by including 1% cetrimide or 100 units/ml penicillin G + streptomycin in the dichromate, or by reducing the pH to the range 1–3. With cetrimide, much material was lost due to foaming, and only 59% of available oocysts were recovered compared with 91% when extraction preceded sporulation. Using penicillin + streptomycin, 83% sporulation and 83% recovery of available oocysts was achieved, compared with 91% recovery and 90% sporulation when extraction preceded sporulation. When the pH was reduced to 1–3 during aeration of the faecal suspension, 76% sporulation was achieved in 72 h at room temperature, but only 53% of the available oocysts could be extracted. No differences in virulence for chickens of sporulated oocysts has been found between those sporulated prior to, and those sporulated after isolation from faeces.

Flotation

Although crude oocyst preparations can no doubt be cleaned up somewhat by repeated washing and sedimentation (Edgar, 1964), we feel the process both time consuming and cumbersome, and prefer to employ flotation.

Choice of medium

Solutions of 58% (w/v) sucrose, 37% (w/v) $ZnSO_4.7H_2O$ and saturated NaCl, which all have sp. grs. of 1·20, were compared for their efficiency in extracting oocysts from dichromate-collected homogenized and sieved faeces (Table 5). Equal amounts of sieved faecal suspension were sedimented in 250 ml centrifuge bottles, resuspended in one of the 3 solutions under test, and then centrifuged (1500 rev./min for 5 min). The oocysts present in the scum which floated were recovered with a syringe fitted with a wide-bore cannula, the residue was resuspended and the volume restored with further saturated NaCl, and a 2nd and a 3rd

centrifugation carried out. Recoveries at each stage with zinc sulphate or sucrose were no better than with common salt, but if anything slightly lower; likewise there was no advantage in using zinc sulphate solutions of sp. gr. 1·30 or 1·40.

Solutions of salt, zinc sulphate and sucrose at sp. gr. 1·20 were compared for their effects on morphology and viability of oocysts which had been quickly isolated from faeces with minimal contact with salt solution. Following 24 h contact at room temperature with saturated NaCl, 69 % of the oocysts showed some degree of deformation and partial collapse of the wall; a few empty collapsed oocyst shells were also noted. After removal of the saturated NaCl by washing, 91 % sporulation was achieved and the oocysts regained their normal shape. Seven days contact with saturated NaCl resulted in a population consisting mostly of collapsed, empty shells; a few unsporulated, but no sporulated oocysts were present. Oocysts in contact with $ZnSO_4$ sp. gr. 1·20 for 24 h showed no abnormalities or deformation, and 8 % had sporulated. After 7 days contact with $ZnSO_4$, 84 % had sporulated, and most oocysts looked normal, although a few showed partial collapse. Oocysts standing in sucrose sp. gr. 1·20 for 24 h showed minor kinking of the wall in 7 % of cases, while a few had started to sporulate. After 7 days contact with sucrose, 63 % sporulation had occurred; some showed normal morphology, some were collapsed. Although marked changes in oocyst shape were most readily produced by saturated NaCl, such effects were reversible on washing following at least 14 h contact, and there was no subsequent inhibition of sporulation. In the case of *E. tenella* at least, there would seem to be no disadvantage in using saturated NaCl as a flotation medium in view of the short periods of contact employed.

Sucrose of sp. gr. 1·20 is viscous and sticky; concentrated solutions can be readily made by heating. Zinc sulphate readily dissolves to give concentrated solutions but it is much more expensive than sucrose. Common salt dissolves slowly, particularly when reaching saturation, and heating helps little (solubility 35·7 % (w/v) at 0 °C, 39·12 % at 100 °C). The most convenient way of preparing a saturated solution is to put water and excess salt in screw-capped bottles (2·5 l), and leave overnight on a roller mill. Because salt is so very much cheaper than either sucrose or zinc sulphate – a consideration in a large-scale operation – and gives rather better recoveries of oocysts with no apparent disadvantages, we recommend flotation with saturated salt as the preferred method of isolation.

Conditions of flotation

Four centrifuge bottles were used to sediment material in 1·6 l sieved faecal homogenate, and 2 flotations in saturated salt were carried out in a total volume of 2 l; 59·8 % of the oocysts were recovered. Twice the volume of the same homogenate was processed in a further 4 bottles, salt flotation once again being carried out in a volume of 2 l. Although more oocysts were obtained from the double quantity of faeces, the recovery rate was only 48 %. In a further experiment, a rather poor recovery rate of 43·4 % was obtained with a certain amount of material, but this was reduced to only 23·3 % when the solid : salt ratio was increased 3-fold.

Three batches of faeces were processed to investigate the effect of oocyst content

Table 6. *Effect of repeated salt flotation on oocyst recovery*

Flotation	% of total oocysts recovered				Average
1	7·9	30·3	9·6	45·1	23·2
2	48·5	32·8	58·5	9·7	37·4
3	16·6	17·4	16·5	10·3	15·2
4	9·2	6·8	6·8	9·7	8·1
5	7·6	5·2	3·9	7·8	6·1
Residue	10·2	7·5	4·7	17·4	10·0

Figures represent % of total oocysts recovered with 5 successive salt flotations in 4 separate experiments, and average % recovery at each stage.

of the faeces on recovery. In each case, 720 g faeces were made to 3 l with water, homogenized, sieved and salt floated; an overall oocyst recovery of 66·8 % was obtained. With each batch, a further 180 g sample of oocyst-containing faeces was mixed with 540 g faeces from uninfected chickens, made to 3 l with water and processed as before. An overall recovery rate of 64·3 % was obtained, suggesting that the actual concentration of oocysts in faeces has little bearing on the efficiency of the extraction procedure.

Table 6 summarizes 4 further experiments in which oocyst recovery was investigated with serial salt flotations. As a result of these experiments, we do not consider that more than 3 flotations are worthwhile when one offsets time and effort against oocysts recovered. Difficulties were also sometimes encountered after 4–5 flotations when a large part of the unwanted debris became flocculent and floated.

Washing

Oocysts removed from the surface of the saturated salt mixtures were squirted into an excess of water – at least 5 volumes were necessary to bring the sp. gr. below 1·03. Although they can be allowed to settle overnight, the supernatant fluid then being decanted, we prefer to recover oocysts by brief centrifugation right away. This means they are no longer in contact with any salt, and can be immediately put in dichromate to sporulate.

Further purification

In preparations isolated as described, oocysts account for only about 50 % of the solids present, the rest being assorted faecal debris. For experiments involving the inoculation of further chickens, this degree of cleanliness is quite adequate, although for some other purposes further purification is necessary. This can be carried out before or after sporulation.

Hypochlorite treatment

For metabolic studies with oocysts or sporozoites, or where bacteriologically sterile sporozoites are required for chick embryo or cell culture inoculation, a

modification of the hypochlorite digestion described by Jackson (1964) is simplest. By using adequate concentrations of hypochlorite, flotation of oocysts and separation from sedimented debris can be achieved. A crude oocyst suspension in 0·8 % (w/v) NaCl is mixed with half its volume of sodium hypochlorite liquor (approx. 30 % (w/v) NaOCl; not less than 14 % available chlorine). After standing at room temp. for 20–30 min, extraneous matter will have dissolved or at least be extensively degraded, and bacteria and fungi will have been killed. The outer layer of the oocyst wall is removed by this procedure (Nyberg & Knapp, 1970), although the viability of the oocyst contents is in no way impaired during this short contact time. Centrifugation with this concentration of salt and hypochlorite gives a pure white scum of oocysts, a virtually clear supernatant, and a sediment containing degraded debris. Three or 4 washes with water or saline are adequate to remove hypochlorite and yield a preparation of oocysts free from any contaminants. Three samples of oocysts were purified as described in the next section, and then subjected to hypochlorite treatment as above. Dry weight determinations indicated that the outer layer of the oocyst wall removed by hypochlorite treatment represented 19·5–21·8 % of the total oocyst dry weight.

Glass bead column and gradient centrifugation

Oocyst purification using continuous (Sharma, Reid & Foster, 1963) or discontinuous (Patnaik, 1966) density gradients have been described. A 250 ml centrifuge bottle was prepared containing layers of equal amounts of 50, 30, 20 and 10 % (w/v) sucrose, and 5 ml concentrated crude oocyst suspension was layered on top. After centrifugation at 1100 *g* for 10 min, oocysts with dirt particles were found floating on the 20 % layer, and a sediment, which contained few oocysts, settled on the bottom of the bottle. No better separation of dirt from oocysts was achieved by including 0·1 M HCl in the gradients. A continuous gradient of 0–50 % sucrose likewise failed to give a pure oocyst fraction. Many oocysts were found in clumps, which also contained small granular aggregated rubbish. These aggregates could be dispersed by suspending the crude oocyst preparation in 5 % Tween 80 and stirring for ½–1 h. Subsequent separation on continuous sucrose density gradients (0–25 %) containing 5 % Tween 80 gave a cleaner – but still impure – oocyst zone. Attempts were made to remove some of the smaller foreign particles by washing on a membrane filter (Sartorius SM 113 01; pore size 8 μm), the suspension being stirred during filtration. However, little purification of the oocysts was achieved. At this stage we started using the glass bead column method of Wagenbach (1969) to separate sporozoites from digests of broken oocysts. Under the conditions described by Wagenbach, oocysts were retained on the column. Using unbuffered 5 % Tween 80, however, we found such columns could be used to effect a preliminary cleaning up of oocysts before gradient centrifugation. A column of glass beads (Superbrite type 100–5005; Minnesota Mining and Manufacturing Co.) 10 cm diameter and 8 cm deep was supported on a sintered glass disk (porosity 3). It was loaded with a crude concentrated oocyst preparation which had been shaken briefly by hand with 7 mm glass beads in 5 % Tween 80 to disperse thoroughly, and washed through with 2 l

of the same medium. Much dirt was retained on the column. The effluent was concentrated to 120 ml by brief centrifugation. Aliquots of 20 ml of this concentrate were layered on continuous sucrose gradients (sp. gr. 1–1·15) in 250 ml centrifuge bottles and spun at 1500 rev./min for 8 min. The oocyst bands which formed partway down the bottles were recovered with a syringe and diluted with 5 vols. water. Final cleaning of the oocysts was achieved by 3 washings with water in the centrifuge, at the slowest speed attainable, for 1 min. This was sufficient to sediment most of the oocysts, but not the residual dirt. The preparation was virtually free from any type of debris or bacteria. An overall yield of 25% of the original oocysts was obtained after both column and gradient purification.

Sporulation

Sporulation is a strictly aerobic process (Wilson & Fairbairn, 1961) which proceeds optimally at a temperature of 29 °C (Edgar, 1954). We have shown earlier that sporulation is preferably carried out subsequent to oocyst isolation, since the bulk of the material requiring aeration is less, and the amount of microbial contamination is reduced (or eliminated with hypochlorite treatment). Using the isolation methods recommended, sporulation can be initiated the same day as the faeces are collected and processed. Sporulation can be achieved in a static system or with forced aeration. Potassium dichromate (2%) is used to discourage microbial growth, although alternative media such as 0·1 M H_2SO_4 (Goodrich, 1944; Wilson & Fairbairn, 1961) are useful.

Static method

Oocysts suspended in 2% potassium dichromate are incubated with free access of air. This is achieved using shallow layers of suspension in trays, dishes or flat-bottomed flasks. We find Glaxo culture vessels (a cylindrical vessel 17·5 cm diameter and 10 cm deep, having a flat bottom and top, and fitted with a side vent) most convenient, since the vessels can be stacked, and can be loosely plugged and sterilized to prevent contamination with extraneous coccidia. Because sporulation is a strictly aerobic process, the critical factors are oocyst concentration and depth of the suspension. Table 7 records an experiment in which 6 different vols. of oocyst suspension made up at 4 different concentrations were incubated for 3 days at 30 °C in Glaxo vessels. It will be seen that better sporulation was achieved in smaller volumes at a given concentration, or lower concentrations at a given volume. Satisfactory sporulation could not be achieved with more than 10^8 oocysts in a single vessel of these dimensions.

Forced aeration

Although sporulation in Glaxo vessels is convenient for small oocyst preparations, large collections would require vast numbers of flasks. The volumes of culture being handled can be appreciably reduced and adequate sporulation obtained if forced aeration is employed. We consider that the oocyst concentration should not exceed 5×10^6 per ml. We routinely aerate such preparations in 500 ml

Table 7. *Sporulation of* Eimeria tenella *under static conditions*

Volume (ml)	Oocyst concentration (millions/ml) 0·25	0·5	1·0	2·0	Depth (mm)
1000	51·7	36·5	19·5	—	41·5
600	81·5	58·5	29·0	—	24·9
400	88·7	77·5	50·0	—	16·6
200	92·8	90·0	86·2	45·5	8·4
100	95·8	90·2	91·7	68·5	4·2
50	95·2	95·0	95·2	84·5	2·1

Figures give % sporulation after incubation in a Glaxo vessel for 3 days at 30 °C. Zig-zag line indicates 10^9 oocysts total.

amounts in 1 l measuring cylinders in an incubator at 30 °C, using a sintered glass disk on the end of an air line immersed to the bottom of the cylinder. With one batch of 500 ml oocysts at 5×10^6 per ml, 92 % sporulation was achieved after 24 h at 30 °C. A 200 ml sample of the same isolate, but diluted to $0{\cdot}5 \times 10^6$ oocysts per ml in a Glaxo vessel reached 73 % sporulation in 24 h, 81 % in 48 h and 94 % in 72 h. Some oocysts accumulate on the walls of the cylinder above the liquid level, but these can be scraped down at intervals.

RECOMMENDED PROCEDURE

Groups of chickens 3–4 weeks of age are caged, and after withholding food overnight, are inoculated with 5000 sporulated oocysts of *E. tenella* (day 0). Food is then supplied *ad lib*. On day 5 of the infection, food is withdrawn, the trays under the cages are cleaned out and 1·5 l 2 % (w/v) aqueous potassium dichromate solution (or water) is added. For the next 3 days, 200 g food per cage is supplied daily. On day 8, the birds are killed, and the 3-day faecal collection in dichromate is homogenized for 3 min with a Vortex mixer. The resulting slurry is passed once through a 40 mesh and then once through a 100 mesh sieve. The slurry is centrifuged for 3 min at 1000 rev./min in 1·5 l plastic bottles, and the supernatant discarded. The sediment is resuspended in saturated NaCl so that the bottles are about two thirds full. After centrifuging again for 3 min at 1000 rev./min, the oocyst-containing scum is removed from the surface by means of a syringe fitted with a wide-bore metal cannula (1·5 mm ID), and is squirted into water. The residue in the centrifuge bottles is resuspended and a second crop of oocysts recovered following centrifugation; this is repeated a third time. The 3 crops of oocysts diluted with water are centrifuged, and the sediment is subsequently made up in 2 % potassium dichromate to a concentration not exceeding 5×10^6 oocysts/ml. The suspension is aerated at 30 °C for 3 days in a measuring cylinder by means of an air line fitted with a sintered glass disk. Oocysts are stored in 2 % potassium dichromate solution

in the refrigerator at 4 °C. The crude oocyst preparation may be further purified if necessary, either by hypochlorite treatment, or by passage through a glass bead column suspended in 5% Tween 80 followed by sucrose density gradient centrifugation.

The work described in this paper was carried out in 1968–1970. Around this time, 48 passages were carried out by the recommended procedure over a 10 month period, yielding 214 l sieved faecal material in dichromate. This contained an initial $1{\cdot}19 \times 10^{11}$ oocysts, and yielded on processing a total of $1{\cdot}0 \times 10^{11}$ oocysts, resulting in an average operating recovery of 84%. The average sporulation rate achieved was 91%. Over the next 5 years, using the Azabu isolate of *E. tenella* and a variation of chick and diet, a total of 100 routine passages were carried out using 7100 chicks 2–4 weeks of age. Starting with a total of 1766 l unsieved faecal suspension containing $4{\cdot}95 \times 10^{11}$ oocysts, sieving reduced the volume to 1437 l and resulted in a loss of 22·7% of the oocysts, with the elimination of much unwanted debris. The final yield of oocysts was $2{\cdot}29 \times 10^{11}$, which represents a recovery rate of close on 60% of the oocysts present in the sieved material, and an overall recovery rate of 46%. Sporulation here has averaged 91·8%. The procedure over this 5 year period has not been entirely constant. The breed of bird has varied, and brooding arrangements available make it necessary at the moment to use birds 2–3 weeks of age rather than a week older. Faecal properties have changed from time to time, apparently correlated with variations in the supposedly standard diet, and oocyst yields have at times fallen dramatically. Better yields of oocysts were obtained at such periods if faeces were collected into water rather than dichromate, though at other times the reverse was the case.

DISCUSSION

The overall concern in our work has been to develop an efficient procedure for oocyst isolation which can be applied on a large scale. Vetterling (1969) with a similar end in view advocated the use of a continuous flow centrifuge and a greater than usual dilution of faeces to avoid sieving. Examination of his recommendations, however, reveals that to process a 3 day output from 25 2- to 4-week-old birds, 36 l 2·5% dichromate and 36 l 2 M sucrose will be required, and a total of 522 l material will have to pass through the centrifuge. Thus, to complete steps 1–4 at 1 l/min will take almost 9 h, not allowing for cleaning the bowl between runs. Several additional hours centrifuging and further quantities of sucrose will be required for steps 5 and 6 (which are done twice over). In fact, from the data given in his Table 1, 36 h would be required to process this amount of material! Using the methods we recommend, a similar quantity of faecal material could be processed in $2\frac{1}{2}$ h, and we would expect to get an overall oocyst extraction of around 50%. Although this recovery is less than half the rather optimistic 106% achieved by Vetterling – in spite of the fact that he had lost the majority of unsporulated oocysts during processing – we consider the saving in time and physical effort more than compensates.

We wish to emphasize that our routine method is not infallible, and details will

have to be varied from time to time to cope with changing circumstances. Nevertheless we believe consideration of the various factors to which we have drawn attention will reduce the burden of a necessary laboratory chore.

A recent paper (Smith & Ruff, 1975) describes an oocyst isolation method which seems to be derived from the well known formol–ether concentration technique used in diagnostic examination of human faeces for a variety of parasitic organisms. Although the method does result in the separation of oocysts, provided the liquid:solids ratio is kept high enough, we would not like to have to use it for what we would call large-scale production. A more detailed appraisal of the technique will form part of a report in preparation.

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